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# Target Region Amplification Polymorphism (TRAP) for Assessing Genetic Diversity in Sugarcane Germplasm Collections

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## ABSTRACT

**Target region amplification polymorphism (TRAP) is a fairly new PCR-based molecular marker technique which uses gene-based information for primer design. The objective of this study was to evaluate the utility of TRAP markers for assessing genetic diversity and interrelationships in sugarcane germplasm collections. Thirty genotypes from the genera *Saccharum*, *Miscanthus*, and *Erianthus* were used in the study. Among the genus *Saccharum* were the species, *S. officinarum* L., *S. barberi* Jesw., *S. sinense* Roxb., *S. spontaneum* L., *S. robustum* Brandes and Jeswiet ex Grassl, cultivars, cultivar-derived mutants and interspecific hybrids between *S. officinarum and S. spontaneum*. Six fixed primers, designed from sucrose- and cold tolerance-related EST (expressed sequence tags) sequences, paired with three arbitrary primers, were used to characterize the germplasm. Both the cluster and principal coordinate (PCoA) analyses placed the *Erianthus* spp. and *Miscanthus* spp. genotypes distinctly from each other and from the *Saccharum* species, thus, supporting their taxonomic classification as separate genera. Genotypes of the low sucrose and cold tolerant species, *S. spontaneum,* formed one distinct group, while the rest of the *Saccharum* species formed one interrelated cluster with no distinct subgroups. Sequence analysis of TRAP bands derived from a *S. spontaneum* genotype revealed homology with known gene sequences from other grass species including *Sorghum*. A BLASTn search using the homologous sequences from *Sorghum* matched with the *S. officinarum* GenBank accession from which the fixed TRAP primer was designed. These results ratify TRAP as a potentially useful marker technique for genetic diversity studies in sugarcane.**

The genus *Saccharum* is composed of six species, namely *S. officinarum* L., *S. barberi* Jesw., *S. sinense* Roxb., *S. spontaneum* L., *S. robustum* Brandes and Jeswiet ex Grassl, and *S. edule* Hassk. (Brandes, 1958). The modern *Saccharum* spp. (cultivated sugarcane) is believed to have originated from complex hybridization events (termed ''nobilization'') between *Saccharum officinarum*, *S. barberi, S. sinense,* and the wild related species *S. spontaneum* (Sreenivasan et al., 1987). Until the end of the 19th century, cultivated sugarcane was comprised mainly of the vegetatively propagated *S. officinarum* (the main sugar producing cane) together with *S. barberi* and *S. sinense* (Jannoo et al., 1999). *Saccharum officinarum*, however, is believed to have evolved through hybridization of species such as *Erianthus arundinaceus* (Retz.) Jeswiet, *S. spontaneum*, and *S. robustum* (Daniels et al., 1975), whereas *S. barberi* and *S. sinense* are believed to be natural hybrids between *S. officinarum* and *S. spontaneum* (Daniels and Roach, 1987). Mukherjee (1957) coined the term *Saccharum* complex to encompass four closely related interbreeding genera viz., *Saccharum, Erianthus* (= sect. Ripidium), *Narenga*, and *Sclerostachya,* all of which are supposedly implicated in the origin of sugarcane. Daniels et al. (1975) revised this grouping to include *Miscanthus* sect. Diandra Keng, but the phylogenetic relationship between members of the group remains unclear (Irvine, 1999).

A better understanding of the genetic diversity and interrelationships among members of the *Saccharum* complex will facilitate exploitation of this germplasm in improving sugarcane. Traditional methods which combined agronomic and morphological characteristics have been useful in identifying and describing differences between members of the *Saccharum* complex (Artschwager and Brandes, 1958; Skinner, 1972; Skinner et al., 1987). However, members of the *Saccharum* complex are predominantly outcrossing and are maintained by vegetative propagation. As such, they are highly heterozygous and display enormous plasticity in the phenotypic expression of traits. Although morphological traits can be used to identify and classify clones, most of the traits are influenced by the environment under which the clones are grown or selected. Variability caused by genotype × environment interactions and inadvertent mislabeling of clones can adversely influence data derived from phenotypic evaluation and clonal records.

With the advent of molecular markers, it is now possible to make direct inferences about genetic diversity and interrelationships among organisms at the DNA level without the confounding effects of the environment and/or faulty pedigree records. Indeed, a vast number of molecular marker techniques such as isoenzymes (Glaszmann et al., 1989), RFLP (restriction fragment length polymorphism) (D'Hont et al., 1994; Jannoo et al., 1999; Coto et al., 2002), ribosomal DNA (Glaszmann et al., 1990; Pan et al., 2000), microsatellites (Piperidis et al., 2001; Cordeiro et al., 2003), AFLP (amplified fragment length polymorphism) (Besse et al., 1998; Lima et al., 2002), and molecular cytogenetics (D'Hont et al., 1996) have been instrumental in explaining genetic diversity and interrelationships among accessions in sugarcane germplasm collections.

The underlying goal for studying genetic diversity and interrelationships among germplasm collections is to eventually use that information to facilitate the development of better performing varieties of the cultivated species. The results from genetic diversity studies may,

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therefore, be more useful if the segments of the genome sampled or measured correspond to segments bearing genes of interest to the breeding program. Current molecular marker tools, such as RFLP, RAPD (random amplified polymorphic DNA), AFLP, and gSSR (SSRs, simple sequence repeats, derived from genomic as opposed to EST sequences), have unarguably been very useful in dissecting the level and pattern of genetic diversity in sugarcane germplasm collections. However, the polymorphism generated by these marker techniques is randomly distributed across the genome and only polymorphism that can be associated with traits through QTL studies would be of immediate interest to the breeder. Even when QTL analysis is performed, the underlying association is often based on relatively large linkage blocks. Transferability of QTLs between populations remains a looming question in the minds of many plant breeders.

Sugarcane remains a complex and recalcitrant crop to study and improve by genetics approaches owing to the large genome size, high ploidy level, and complicated genome organization. However, recent access to increasing numbers of sugarcane EST sequences obtained from diverse cDNA libraries coupled with freely available bioinformatics tools now allow us to explore new opportunities in sugarcane molecular marker research. TRAP is a simple PCR-based marker technique which uses EST or gene information to generate polymorphism (Hu and Vick, 2003). A fixed primer of about 18 nucleotides is designed from EST sequences or genes of interest and an arbitrary primer of about the same length is designed with either an AT- or GC-rich motif to anneal with an intron or exon, respectively (Hu and Vick, 2003; Li and Quiros, 2001). TRAP markers have not previously been used to genotype sugarcane. Therefore, the objective of this study was to evaluate the potential of TRAP markers for assessing genetic diversity in sugarcane germplasm collections.

## MATERIALS AND METHODS

### Plant Material and DNA Extraction

Thirty genotypes, representing three genera namely *Saccharum*, *Miscanthus*, and *Erianthus* were used in the study (Table 1). Representing *Saccharum* species were *S. officinarum, S. barberi*, *S. sinense, S. spontaneum, S. robustum*, as well as cultivars, cultivar-derived mutants, and interspecific hybrids. The genotypes Dwarf 1 and Dwarf 2 are cultivar-derived genetic mutants from the cultivar LCP81–137 (Burner, 1999). The Genotypes 16 Low and 40 High are first generation interspecific hybrids from a cross between La Stripe (*S. officinarum*) × SES 147b (*S. spontaneum*) and are being retained in the collection because of their low and high sucrose content, respectively. These genotypes form part of the germplasm collection maintained at the USDA Sugarcane Research Unit at Houma, LA. Young leaves were collected from each genotype, frozen immediately in ice and stored at −80 C. The leaves were later ground to a powder in liquid nitrogen. Genomic DNA was extracted with the Plant DNeasy Mini Kit (Qiagen, Valencia, CA) following the manufacturer's protocol. Concentrations of extracted DNA were estimated by known concentration of Lambda DNA in 1% (w/v) agarose gel.

**Table 1. Description of the 30 genotypes used for TRAP marker analysis.**

| Number | Clone name | Genera or species† | Code |
|---|---|---|---|
| 1 | Kalingpong | *Erianthus* | Er |
| 2 | Dwarf 1 | *Saccharum* species hybrid (cultivar-derived mutant) | DW1 |
| 3 | Dwarf 2 | *Saccharum* species hybrid (cultivar-derived mutant) | DW2 |
| 4 | 16 Low | *Saccharum* species hybrid ($F_1$ between *S. officinarum and S. spontaneum*) | Hy1 |
| 5 | 40 High | *Saccharum* species hybrid ($F_1$ between *S. officinarum and S. spontaneum*) | Hy2 |
| 6 | POJ2878 | *Saccharum* species hybrid (cultivar) | Cu1 |
| 7 | LCP 85–384 | *Saccharum* species hybrid (cultivar) | Cu2 |
| 8 | CP 77–310 | *Saccharum* species hybrid (cultivar) | Cu3 |
| 9 | CP 77–407 | *Saccharum* species hybrid (cultivar) | Cu4 |
| 10 | HoCP 85–845 | *Saccharum* species hybrid (cultivar) | Cu5 |
| 11 | Miscanthus | *Miscanthus* | Mi |
| 12 | Ganapathy | *S. barberi* | Sb1 |
| 13 | Chin | *S. barberi* | Sb2 |
| 14 | La Stripe | *S. officinarum* | So1 |
| 15 | La Purple | *S. officinarum* | So2 |
| 16 | Cuba | *S. officinarum* | So3 |
| 17 | IN84–068A | *S. officinarum* | So4 |
| 18 | NG 57–54 | *S. robustum* | Sr1 |
| 19 | NG 57–159 | *S. robustum* | Sr2 |
| 20 | Molokai 5573 | *S. robustum* | Sr3 |
| 21 | IMP72–232 | *S. robustum* | Sr4 |
| 22 | NG77–218 | *S. robustum* | Sr5 |
| 23 | Chukche | *S. sinense* | Ssi |
| 24 | SES 147b | *S. spontaneum* | Ssp1 |
| 25 | Coimbatore | *S. spontaneum* | Ssp2 |
| 26 | MPTH97–213 | *S. spontaneum* | Ssp3 |
| 27 | MPTH97–200 | *S. spontaneum* | Ssp4 |
| 28 | MPTH97–107 | *S. spontaneum* | Ssp5 |
| 29 | PIN84–1B | *S. spontaneum* | Ssp6 |
| 30 | Molokai1032B | *S. spontaneum* | Ssp7 |

† The original sugarcane cultivars were derived from crossing mainly between *S. officinarum and S. spontaneum* followed by several generations of backcrosses to *S. officinarum.* Present day cultivars are selections from cultivar × cultivar crosses.

### TRAP Markers

#### Primer Design

The design of fixed primers was based on the method reported by Hu and Vick (2003). The nucleotide sequences of six genes of interest were obtained from the GenBank database at NCBI. Of the six selected genes, five are believed to be involved in carbohydrate (sucrose) metabolism, while the remaining one is believed to play an important role in cold tolerance. The primers were designed by the Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi; verified 15 September 2005) (Rozen and Skaletsky, 2000), out of which only the forward primer was used as a fixed primer. The primer optimum size, maximum size and minimum size were set to 18 nt. The optimum $T_m$, maximum $T_m$, and minimum $T_m$ were set to 53, 55, and 50°C, respectively. The genes, GenBank accession numbers, and designed primer sequences used in this study are given in Table 2.

Arbitrary reverse primer sequences were obtained from Li and Quiros (2001). These primers comprise three selective nucleotides at the 3′ end, 4 nucleotides of AT- or GC-rich content in the core region and 11 nucleotides as filler sequences at the 5′ end. In addition, the basic rules of primer design such as self-complementarity and maintenance of 40 to 60% GC content were upheld (Table 2). The AT- and GC-rich

**Table 2. Sequences of fixed and arbitrary primers used for TRAP markers.**

| | Gene | Sequence (5′→ 3′) | GenBank accession number |
|---|---|---|---|
| **Fixed primers** | **Sucrose synthase (SuSy)** | **GGAGGAGCTGAGTGTTTC** | **AF263384** |
| | **Sucrose phosphate synthase (SuPS)** | **CGACAACTGGATCAACAG** | **AB001338** |
| | **Pyruvate orthophosphate dikinase (PODK)** | **CGTAAAGATTGCTGTGGA** | **AF194026** |
| | **Soluble acid invertase (SAI)** | **AGGACGAGACCACACTCT** | **AF062735** |
| | **Calcium dependent protein kinase (CDPK)** | **ACAGAACCACCAAAGGAG** | **CF572977** |
| | **Starch synthase (StSy)** | **GGCAAGAAGAAGTTCGAG** | **AF446084** |
| **Arbitrary primers** | **Arbi 1** | **GACTGCGTACGAATTAAT** | |
| | **Arbi 2** | **GACTGCGTACGAATTGAC** | |
| | **Arbi 3** | **GACTGCGTACGAATTTGA** | |

primers supposedly target introns and exons, respectively (Li and Quiros, 2001).

### PCR Protocol

TRAP reactions were performed on the basis of the protocol of Hu and Vick (2003). Fixed primers were combined with each of three arbitrary primers for a total of 18 primer combinations. Each reaction was performed in a total volume of 20 μL containing 1.5 μL of 10× PCR buffer, 1.0 μL of 25 m*M* $MgCl_2$, 1.0 μL each of 10 μM fixed and arbitrary primers, 1.0 μL of 10 μM dNTPs (Promega, Madison, WI), 0.35 μL of 5U *Taq* polymerase (Promega, Madison, WI) and 1.0 μL of 50 to 80 ng genomic DNA. The conditions for PCR were as follows: an initial denaturing step was performed at 94°C for 4 min followed by 5 cycles at 94°C for 45 s, 35°C for 45 s and 72°C for 1 min, followed by 35 cycles at 94°C for 45 s, 53°C for 45 s, and 72°C for 1 min with a final extension step at 72°C for 7 min. All the PCR reactions were performed on an *i-cycler* (BioRad Labs, Hercules, CA). After PCR, the amplified products were run on 7% (w/v) polyacrylamide denaturing gel for 2.0 h at 110 W. Silver staining procedure was employed to develop the gel and to detect the bands.

### Statistical Analysis

Bands from the TRAP gel were scored, as "1" for presence and "0" for absence, in all genotypes. Only readable bands were scored, while ambiguous bands were ignored and excluded from the analysis. Allelic diversity at a given locus can be measured by Polymorphism information content (PIC), wherein a marker can distinguish two alleles taken at random from a population, and it was calculated as follows:

$$\text{PIC} = 1 - \Sigma f_i^2$$

where, $f_i$ is the frequency of the $i$th allele (Weir 1990). Considering the number of alleles at a locus along with their relative frequencies in a given population, an estimate of the discriminatory power of a marker can be obtained by calculating the PIC (Vuylsteke et al., 2000). Jaccard-similarity coefficient (1908) was used to calculate the estimate of genetic similarity (GS) among pairs of genotypes as follows:

$$\text{GS}_{ij} = a/(a + b + c)$$

where $\text{GS}_{ij}$ is the genetic similarity measurement between individuals $i$ and $j$, the number of polymorphic bands present in both individuals is represented by $a$, whereas $b$ and $c$ are the number of bands present in individual $i$ and $j$, respectively, but not in their counterparts. The GS matrix was used to perform cluster analysis using the Unweighted Pair Group Method with Arithmetic mean (UPGMA) algorithm (Sneath and Sokal, 1973) following the Sequential Agglomerative Hierarchical Nested (SAHN) cluster analysis. The cophenetic values were calculated to test the goodness of fit between the clusters in the dendrograms and the similarity index matrix. In addition, PCoA was performed to supplement the findings obtained from cluster analysis. All the above analyses were performed employing different modules of NTSYS-PC software, version 2.11L (Rohlf, 2000). For the purpose of comparison between clusters and also to determine the robustness of the cluster, bootstrap analysis was done with 10 000 replications using the PAUP version 4.0b10 software (Sinauer Associates Inc., MA), which employs Nei and Li (1979) method for cluster development.

## RESULTS AND DISCUSSION

### TRAP Marker Polymorphism and PIC Values

The summary of TRAP markers produced by the 18 primer combinations (six fixed forward primers in combination with three arbitrary reverse primers) across all 30 genotypes is given in Table 3. The 18 primer combinations generated a total of 600 unambiguous bands of which 529 (88%) were polymorphic. The bands ranged in size from 100 to 700 bp. The number of bands detected by individual primer combinations ranged from 15 (SuSy + Arbi 3) to 58 (CDPK + Arbi 3) with an average of 33. These two primer combinations were also responsible for the least (14 in SuSy + Arbi 3) and the most (48 in CDPK + Arbi 3) number of polymorphic bands produced for an average of 29 polymorphic bands per primer combination. Polymorphism was generally high (>50%), ranging from 72 to 100%.

**Table 3. Total number of bands, number of polymorphic bands, percent polymorphism and polymorphism information content (PIC) for each of 18 TRAP primer combinations.**

| Primer combination | Bands observed | Polymorphic bands | Percentage polymorphism | PIC value |
|---|---|---|---|---|
| **SuSy + Arbi 1** | **20** | **20** | **100.00** | **0.32** |
| **SuSy + Arbi 2** | **32** | **32** | **100.00** | **0.28** |
| **SuSy + Arbi 3** | **15** | **14** | **93.33** | **0.20** |
| **SuPS + Arbi 1** | **19** | **17** | **89.47** | **0.33** |
| **SuPS + Arbi 2** | **48** | **47** | **97.91** | **0.24** |
| **SuPS + Arbi 3** | **29** | **21** | **72.42** | **0.14** |
| **SAI + Arbi 1** | **39** | **34** | **94.87** | **0.26** |
| **SAI + Arbi 2** | **28** | **28** | **100.00** | **0.22** |
| **SAI + Arbi 3** | **46** | **40** | **86.95** | **0.21** |
| **StSy + Arbi 1** | **41** | **31** | **75.60** | **0.20** |
| **StSy + Arbi 2** | **50** | **41** | **82.00** | **0.24** |
| **StSy + Arbi 3** | **28** | **28** | **100.00** | **0.21** |
| **PODK + Arbi 1** | **36** | **31** | **86.11** | **0.27** |
| **PODK + Arbi 2** | **29** | **22** | **75.86** | **0.36** |
| **PODK + Arbi 3** | **32** | **27** | **84.37** | **0.29** |
| **CDPK + Arbi 1** | **29** | **29** | **100.00** | **0.11** |
| **CDPK + Arbi 2** | **21** | **19** | **90.47** | **0.25** |
| **CDPK + Arbi 3** | **58** | **48** | **82.75** | **0.23** |
| **Total** | **600** | **529** | | |
| **Average** | **33.33** | **29.38** | **88.14** | **0.24** |

The high level polymorphism could be attributed to the complex genetic structure of sugarcane with high (aneu) ploidy comprising 80 to 140 homo(eo)logous chromosomes. Similar high levels of polymorphism have been reported in *Saccharum* species by Besse et al. (1998) and Lima et al. (2002) using AFLP markers. The polymorphism information content (PIC), which measures information content as a function of a marker system's ability to distinguish between genotypes (Weir, 1990), varied among the primer combinations ranging from 0.11 in CDPK + Arbi 1 to 0.36 in PODK + Arbi2 with an average of 0.24. The PIC values indicate a good discriminatory power of the dominant TRAP marker system. Comparable PIC values have been reported with dominant markers like RAPD and AFLP in African plantain (Ude et al., 2003) and AFLP in wheat (Bohn et al., 1999).

For the 18 TRAP primer combinations, the Jaccard's GS estimates ranged from 0.33 (Kalingpong and *Miscanthus*) to 0.94 (Dwarf 1 and Dwarf 2) with a mean of 0.68. A dendrogram with a cophenetic value of 0.96 was generated (Fig. 1) on the basis of 435 pair-wise GS estimates. A cophenetic value of $> 0.80$ is said to indicate a strong goodness of fit for dendrograms (Rohlf, 2000). Bootstrap, determined on the basis of 10 000 resamplings of the data set, and cluster analyses following the Nei and Li (1979) method produced a similar dendrogram (data not shown). This further confirmed the robustness of the dendrogram obtained by the UPGMA method based on Jaccard's similarity coefficient (Fig. 1).

## Genetic Diversity and Relationships among Genotypes

Genetic diversity and relationships among the genotypes in this study were depicted by both cluster and PCoA (Fig. 1 and 2). A separate analysis was performed for the three cold tolerance-related primer combinations (i.e., CDPK/Arbi1, 2, and 3). Because the results did not differ from the one derived from the 15 sucrose-related primer combinations, the data were merged and used for one combined analysis. The dendrogram from cluster analysis revealed two distinct groups among the *Saccharum* species. Group I comprised the genotypes representing *S. officinarum*, *S. sinense, S. barberi, S.*

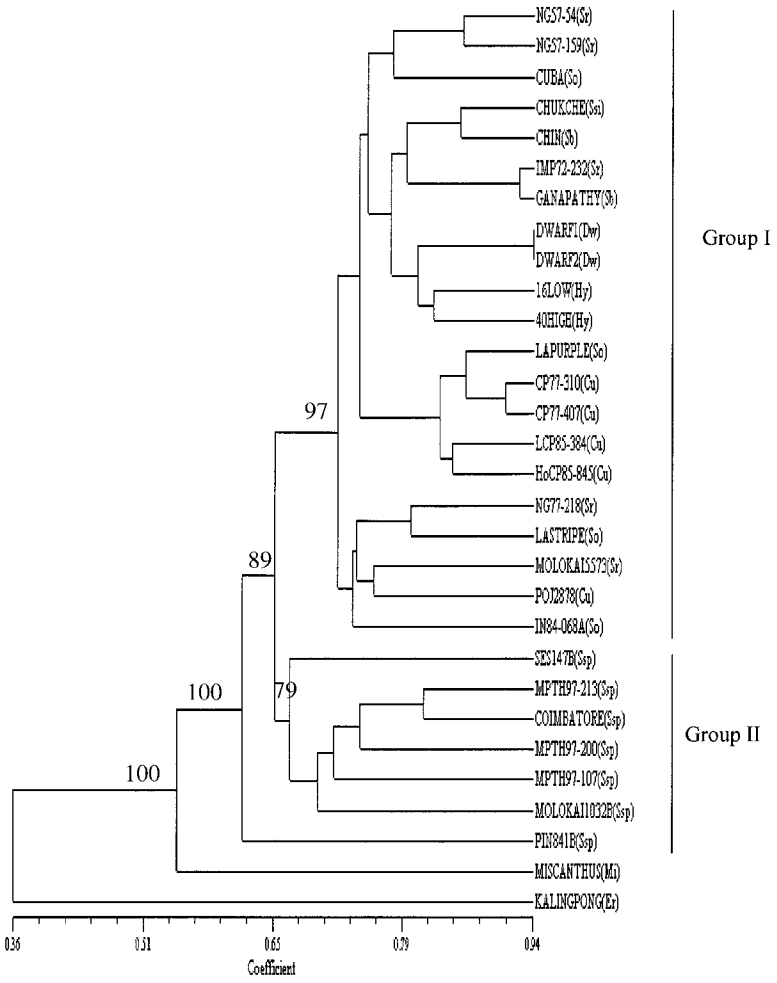


**Fig. 1. Grouping among 30 genotypes from a sugarcane germplasm collection based on 18 TRAP primer combinations using the UPGMA method. Numbers represent values from boostrap analysis. Abbreviations: Mi, *Miscanthus*; Er, *Erianthus*; Cu, Cultivar; Ssp, *Saccharum spontaneum*; So, *S. officinarum;* Sr, *S. robustum*; Sb, *S. barberi*; Ssi, *S. sinense*; Hy, Hybrid; Dw, Dwarf.**

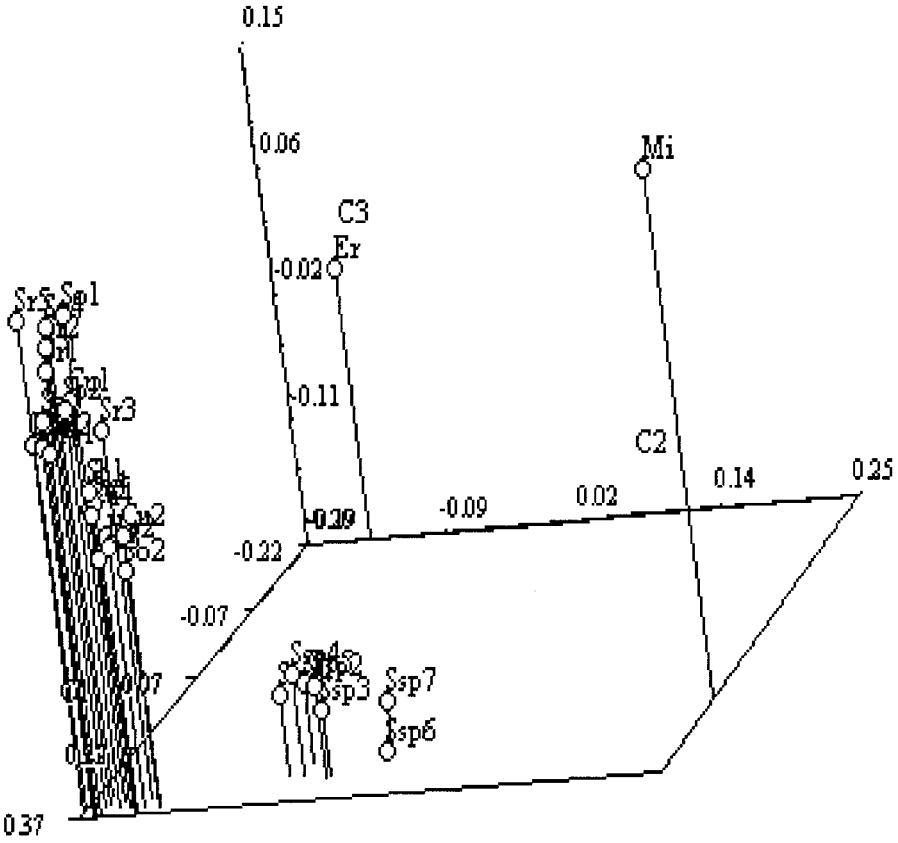


**Fig. 2. Association among 30 genotypes from a sugarcane germplasm collection as revealed by PCoA of genetic distances based on 18 TRAP primer combinations. Abbreviations: Mi, *Miscanthus*; Er, *Erianthus*; Cu, Cultivar; Ssp, *Saccharum spontaneum*; So, *S. officinarum;* Sr, *S. robustum*; Sb, *S. barberi*; Ssi, *S. sinense*; Hy, Hybrid; Dw, Dwarf. Numbers were used to uniquely identify a genotype (for example Ssp6) when there was more than one genotype representing a species or group.**

*robustum* along with cultivars, cultivar-derived mutants, and hybrids, while Group II comprised all the *S. spontaneum* genotypes. The single *Erianthus* (Kalingpong) and *Miscanthus* genotypes were each placed distinctly in the dendrogram, supporting the taxonomic evidence which assigned each of them to a separate genus (Daniels et al., 1975). Remarkably similar results were obtained from the PCoA (Fig. 2). The first three axes in the PCoA explained a cumulative variation of 42.23%. As with the cluster analysis, all the *S. spontaneum* genotypes formed a well individualized group, while the rest of the *Saccharum* species along with the cultivars, cultivar-derived mutants, and hybrids clustered together as one interrelated group. *Miscanthus* and *Erianthus* (Kalingpong) were placed distinctly, again lending credence to the claim that they are separate genera.

The strong differentiation between *Erianthus* and *Saccharum* as revealed by TRAP markers was previously demonstrated with rDNA spacers (Al-Janabi et al., 1994), RFLP (Burnquist et al., 1992), AFLP (Besse et al., 1998), 5S RNA intergenic spacers (Pan et al., 2000), and sugarcane- (Cordeiro et al., 2003) as well as maize- (Selvi et al., 2003) derived microsatelite markers. Similarly, evidence from microsatellite markers (Cordeiro et al., 2003) and 5S RNA intergenic spacers (Pan et al., 2000) had previously been used to document the distinction between *Miscanthus* and *Saccharum* species.

Although no distinct subgroups were found within Group I, the clustering of genotypes in this group seems to be in accordance with the ancestral relationships among these species (Fig. 1). *Saccharum robustum* is believed to be one of the progenitors of *S. officinarum* (Brandes, 1958; Daniels and Roach, 1987). Significant similarities have been reported between *S. robustum* and *S. officinarum* with regards to morphology, cytology, and physiology; however, they differed in fiber and sugar content (Irvine, 1999). The high degree of similarity between *S. robustum* and *S. officinarum* has also been revealed by RAPD (Nair et al., 1999) and microsatellite markers derived from maize (Selvi et al., 2003).

*Saccharum barberi* and *S. sinense* genotypes were found within the same subgroup albeit along with a *S. robustum* genotype. *Saccharum barberi* and *S. sinense* might differ enough to be distinct (Glaszmann et al., 1990), but Whalen (1991) contends that those minor differences are not enough to classify them as two separate species. Moreover, these two species are thought to be interspecific hybrids between *S. officinarum* and *S. spontaneum* (Brandes, 1958; Daniels and Roach, 1987), and this has been substantiated by evidence from RFLP (Lu et al., 1994), RAPD (Nair et al., 1999), and maize-derived microsatellite markers (Selvi et al., 2003).

It was also not surprising that cultivars were found in Group I, indicating their closer relationship with

*S. officinarum* compared with *S. spontaneum.* Most of the cultivars bred after the turn of the 20th century are interspecific hybrids between *S. officinarum* and *S. spontaneum.* However, cultivars inherited a greater proportion of the *S. officinarum* genome as nobilization involved several backcrosses to the *S. officinarum* parent during which this parent transmitted the somatic chromosome number to its progeny (Bhat and Gill, 1985; Bremer, 1961; Sreenivasan et al., 1987; D'Hont et al., 1996).

The closest relationship in the dendrogram was found between the two cultivar-derived dwarf mutants, which is in agreement with the origin of these genotypes. Except for the legendary cultivar POJ2878, all the contemporary cultivars were found in the same subgroup, albeit with a *S. officinarum* genotype. This is hardly surprising since these contemporary cultivars are more closely related relative to POJ2878. However, it was interesting to note that within this subgroup, LCP85–384 clustered closer to HoCP85–845 than it did to either of its parents, namely CP77–310 (female), and CP77–407 (male). LCP85–384 and HoCP85–845 share a common heritage in that their grand parents are full siblings. But the closer association between these genotypes, relative to that between LCP85–384 and its parents, is possibly due to the effects of breeding and selection which is not accounted for by pedigree history. Furthermore, the primers employed in this study were designed to preferentially amplify a small segment of the genome, that is, segments associated with sucrose content and cold tolerance. The effect of selection, especially for sucrose related genes, coupled with the fact that only a small portion of the genome was being assayed, could perhaps explain why the genotypes in Group I failed to form distinct subgroups and clustered instead as one interrelated group. In a study using maize-derived microsatellite markers, *S. barberi* and *S. sinense* genotypes grouped together, but the group was placed in between the *S. officinarum* and *S spontaneum* clusters (Selvi et al., 2003). The authors used this as evidence to suggest that both *S. officinarum* and *S. spontaneum* were involved in the ancestry of these two species.

The average GS within and among groups of genotypes or species was computed as an additional measure to assess genetic diversity (Table 4). Only groups or species represented by at least four genotypes were considered. The estimates showed that the least amount of similarity existed among the *S. spontaneum* genotypes (0.68), indicating the relatively higher level of heterozygosity and polymorphism that exist within this species. *Saccharum spontaneum* is generally accepted as the most diverse of the *Saccharum* species in terms of geographical distribution, chromosome number ($2n$ = 40–128), and morphology (Daniels and Roach, 1987). *Saccharum spontaneum* is considered an untapped resource for sugarcane germplasm improvement in Louisiana. The major focus has been on traits such as disease resistance, cold tolerance, and ratooning ability, although recent evidence using molecular markers suggest that wild relatives such as *S. spontaneum* (with relatively low sucrose content) cannot be discounted as potential contributors of novel genes for traits such as sucrose content (Tanksley and McCouch, 1997; Reffay et al., 2005). TRAP markers could potentially be useful for identifying novel variation and for introgression breeding.

**Table 4. Mean genetic similarity (GS) within and between *Saccharum* species.**

| | *S. officinarum* | *S. spontaneum* | *S. robustum* | Cultivars |
|---|---|---|---|---|
| ***S. officinarum*** | **0.71†** | | | |
| ***S. spontaneum*** | **0.62** | **0.68** | | |
| ***S. robustum*** | **0.74** | **0.63** | **0.76** | |
| **Cultivars** | **0.74** | **0.66** | **0.73** | **0.80** |

**† The numbers in the diagonal are for "within" GS estimates.**

Compared with *S. spontaneum*, genetic similarity was higher among *S. officinarum* < *S. robustum* < cultivars. Genetic diversity has generally been reported as being very low among cultivated sugarcane as very few progenitor clones were involved in the initial 'nobilization' event and the products from nobilization became the foundation clones for most breeding programs.

The highest genetic similarity among groups was obtained between cultivars and *S. officinarum* (0.74) followed by *S. robustum* and *S. officinarum* (0.74). These results provide additional support that, *S. robustum* is a likely progenitor of *S. officinarum* (Sreenivasan et al., 1987) and that cultivars inherited most of their sucrose related genes from *S. officinarum.* The least amount of similarity was observed between *S. officinarum* and *S. spontaneum* (0.62), reflecting the distinctness of these two species.

## Genus and/or Species Specific Markers

Generally, very few bands were discrete across species or genus. The main types of uniqueness found were situations where a band was either present or absent among all genotypes of a species; but, the same band was polymorphic among the other species or genotypes. For example, whereas a SuSy + Arbi 2 (500–600 bp) fragment was polymorphic among *S. spontaneum* and cultivars, this fragment was uniquely absent in all the *S. officinarum, S. robustum, S. barberi*, *S. sinense*, *S. officinarum* × *S. spontaneum* hybrids, and cultivar-derived dwarf genotypes and present in the two *Erianthus* and *Miscanthus* genotypes. Fragment SuSy + Arbi 3 (350–400 bp) was absent in all *S. spontaneum, Erianthus*, and *Miscanthus* genotypes and present among the rest of the genotypes except among cultivars where it was present in three of the five genotypes. But the most significant fragment was SuPS + Arbi1 (600–700 bp), which was present in the two *Erianthus* and *Miscanthus* genotypes but more importantly in all the cultivars and *S. officinarum* genotypes and absent in all *S. spontaneum* genotypes. Fragments which are unique to either *S. officinarum* or *S. spontaneum* are of significance to the Louisiana introgression breeding program because *S. officinarum* and *S. spontaneum* are being used as sources of genes to increase sucrose content and stress tolerance. Another interesting fragment was produced by SAI + Arbi 2 (600–700 bp), which was absent among all the

*Saccharum* species and present in the two *Erianthus* and *Miscanthus* genotypes. Such a fragment could be useful in distinguishing *Saccharum* from other genera.

### Sequencing of Amplified Products

TRAP amplicons were sequenced in an effort to verify if indeed the TRAP marker binding sites correspond to candidate genes. The bands were excised from a PAGE gel, re-amplified and sequenced directly. We sequenced bands from SES 147b a *S. spontaneum* genotype. A fragment of 535 bp from *S. spontaneum,* amplified by the StSy + Arbi3 primer combination, showed homology (E = 5.7) with an EST (Accession # AF079258) of a *Sorghum bicolor* granule-bound starch synthase gene. A similar level of homology (E = 5.7) was found with the ESTs of the granule-bound starch synthase genes of *Cymbopogon pospischilii* (Accession # AF079248), *Heteropogon contortus* (Accession # AF079253) and *Coelorachis selloana* (Accession # AY062271.1). Much higher levels of homology were found with the mRNA sequence of *Zea mays* endosperm transcriptome (E = 6e-37; Accession # BT018673.1) and a cDNA clone corresponding to chromosome 3 of *Oryza sativa* (E = 2e-15; Accession # AK105342.1).

We undertook further analyses of the sorghum EST sequence because sorghum is considered a relative of sugarcane on the basis of comparative mapping studies (Paterson et al., 1995; Ming et al., 1998; Ming et al., 2002). The sorghum EST sequence showed a 100% match with a segment of the 535-bp sequence from *S. spontaneum*. A BLASTn search using the sorghum sequence pulled up, among other sequences, the same EST of *Saccharum officinarum* (Accession # AF446084) from which the "StSy" fixed primer was designed.

Another fragment of 295 bp from *S. spontaneum,* amplified by the CDPK + Arbi2 primer combination showed homology (E = 0.19) with a segment of mRNA corresponding to a putative receptor-like protein kinase gene of *Oryza sativa.* In addition, it showed homology (E = 0.19) with a segment of a clone from chromosome 5 of *Oryza sativa* containing a putative receptor-like protein kinase gene. There is an obvious need for further sequencing analyses using more than one genotype to demonstrate that similar-sized TRAP bands are allelic.

## CONCLUSIONS

Our results provide support for the utility of TRAP markers for assessing genetic diversity in sugarcane germplasm collections. TRAP primers are designed from ESTs or gene sequences; thus, the potential to generate polymorphism around targeted gene sequences is an attractive feature of TRAP markers. Although the TRAP markers reported here have yet to be mapped in sugarcane, results from sequencing and BLASTn analyses of TRAP amplicons lend some support to the proposal that TRAP primers may be targeting gene regions. This was further substantiated by the aggregation of genotypes in the study which seemed to reflect the fact that the TRAP primers were designed from sucrose- and cold tolerance-related gene/EST sequences. Genotypes of the relatively low sucrose and cold tolerant species, *S. spontaneum,* formed one distinct group. Whereas, in contrast to previous studies, genotypes of the high-sucrose and cold-sensitive *Saccharum* species including S. *officinarum, S. barberi*, *S. sinense,* and *S. robustum,* formed one interrelated cluster with no distinct subgroups. TRAP markers could potentially be useful in the characterization and management of domesticated and wild germplasm where the aim is to enhance the germplasm for specific traits. Genetic diversity could be evaluated by TRAP markers for the trait(s) of interest and genotypes or species displaying unique diversity selected for germplasm enhancement. We are currently employing TRAP markers in a QTL mapping study in an effort to further authenticate their potential to target gene regions.

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